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DURING PREGNANCY

For the degree of Master of Science

Is approved by the final examining committee:

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Approved by Major Professor(s): Guoli Dai

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Head of the Department Graduate Program Date

LINEAGE TRACING OF *ASCL1*-EXPRESSING CELLS IN THE MATERNAL LIVER DURING PREGNANCY

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of

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by

Shashank Manohar Nambiar

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LIST OF ABBREVIATIONS

4-OHT	4-Hydroxytamoxifen
Ascl1	Achaete-scute Homolog 1
bHLH	Basic Helix-Loop-Helix
Cre	Cre-recombinase Enzyme
CreER	Cre-recombinase Estrogen Receptor
DAB	3, 3-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DOX	Doxycyclin
DPBS	Dulbecco's Phosphate Buffered Saline
F1	First Filial
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
HNF4α	Hepatocyte Nuclear Factor 4 Alpha
HSCs	Hepatic Stellate Cells
LacZ	β-Galactosidase Enzyme
LPCs	Liver Progenitor Cells
NIH	National Institute of Health
NP	Non-Pregnant
OCT	Optimal Cutting Temperature Medium
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RS26	Rosa26 Ubiquitous Promoter
rtTA	Reverse Tetracycline-Controlled Transactivator
Ttr	Transthyretin
wt	Wild Type
X-Gal	X-Galactopyranoside

ABSTRACT

Nambiar, Shashank M. M.S., Purdue University, August 2014. Differentiation and regulation of *Ascl1*-expressing cells in the maternal liver during pregnancy. Major Professor: Guoli Dai.

To cope with the high metabolic demands of the body during pregnancy, the maternal liver adapts by increasing its mass and size. This increase is proportional to the increase in total body weight during the course of gestation. The pregnancy-induced maternal liver growth is a result of both hepatocyte hypertrophy and hyperplasia. Microarray analysis of pregnant maternal livers shows markedly different gene expression profiles when compared to a non-pregnant state. Most interesting was the 2,500-fold up-regulation in the mRNA expression of *Ascl1*, a transcription factor responsible for the differentiation of neural progenitor cells into various neuronal types, during the second half of pregnancy. Our investigation aimed at (1) characterizing the identity of maternal hepatic *Ascl1*-expressing cells and (2) tracing the fate of *Ascl1*-expressing cells in the maternal liver during pregnancy. Timed pregnancies were generated and non-pregnant (NP) and pregnant maternal livers were harvested and analysed. To identify the maternal hepatic *Ascl1*-expressing cells we used the $Ascl1^{GFP/+}$ reporter mouse line. NP and gestation day 15 (D15) maternal livers were immunostained for green fluorescent protein (GFP). The result shows that GFP-positive, *Ascl1*-expressing cells are hepatocyte-like cells, which

are present in D15 maternal livers, but absent in NP livers. The Rosa26$^{floxstopLacZ/floxstopLacZ}$;Ascl1$^{CreERT2/+}$ mouse line was used to trace the fate of *Ascl1*-expressing cells during pregnancy. LacZ staining of gestation day 13 (D13) and 18 (D18) maternal livers demonstrates that D13 hepatic *Ascl1*-expressing cells (labeled with LacZ) undergo hyperplasia to repopulate a large portion of D18 maternal livers. Furthermore, LacZ and HNF4α co-staining of D13 and D18 maternal livers shows the presence of two populations of LacZ-expressing cells: HNF4α^+ population and HNF4α^- population. HNF4α^+ LacZ-expressing cells represent hepatocyte lineage cells that are derived from *Ascl1*-expressing cells. We observe that, towards the end of pregnancy, a considerable portion of the maternal liver is comprised of hepatocytes derived from *Ascl1*-expressing cells. Taken together, our preliminary study suggests that pregnancy induces maternal liver turnover via *Ascl1*-expressing cells.

CHAPTER 1 INTRODUCTION

1.1 Objectives

The objectives of the study were to characterize and trace the lineage of *Ascl1*-expressing cells in the maternal liver during the course of pregnancy.

1.2 Liver

The liver is a reddish brown coloured and multi-lobed organ that lies in the abdominal cavity, just below the right lung, opposite the stomach. Weighing around 1.4 to 1.6 kg on average, it is the largest internal organ and the largest gland in the human body. The liver performs a myriad of functions. It is responsible for the production and secretion of various substances such as plasma proteins, various lipoproteins, bile, hormones, etc. It detoxifies most of the metabolic waste products as well as neutralizes various xenobiotic substances that the body ingests. It processes the dead red blood cells and recycles many of its components. In addition, the liver is a major site for carbohydrate and fat metabolism and acts as a silo for storing a number of substances such as glycogen, fat, and vitamins. In short, the liver is the pivotal organ for maintaining metabolic homeostasis.

The cells in the liver can be classified into two broad categories: parenchymal cells and non-parenchymal cells. Parenchymal cells, also known as hepatocytes, represent 60% of the total hepatic cells that make-up around 80% of total liver mass. They are responsible for performing most of the above mentioned functions. Non-parenchymal cells include biliary epithelial cells lining the bile ducts, Kupffer cells responsible for phagocytosis of unwanted foreign particles, sinusoidal endothelial cells lining the liver sinusoids, various types of lymphocytes, and hepatic stellate cells for vitamin A storage (Friedman, 2008). An arrangement of these cells along with the afferent and efferent blood vessels and bile ducts form the hepatic lobule. The hepatic lobule is the basic structural unit of the liver, which is shaped roughly like a hexagon. It is comprised of a centrally located central vein that is surrounded by six portal triads representing the vertices of the hexagonal lobule. The portal triad comprises the bile duct, the portal vein, and the hepatic artery grouped together with dense connective tissue. Blood from the portal vein and hepatic artery of each portal triad drains into the central vein via hepatic sinusoids that converge towards the centre of the hepatic lobule. Rows of hepatocytes radiate outwards towards the periphery of the lobule and receive blood flowing through the sinusoids into the central vein. The region between the hepatocytes and the fenestrated endothelium of the sinusoids is called the space of Disse. This space is occupied by resident hepatic stellate cells. Hepatocytes, producing bile, line the bile canaliculi. Bile is released into this channel that then flows through a short and narrow passage called the canal of Hering and into the bile duct. Biliary epithelial cells line the canal of Hering and bile duct. Liver stem cells, also called oval cells, reside in the region

around the bile duct and the canal of Hering (Friedman, 2008; Duncan et al., 2009; Mall., 1906; Michalopoulos, 2013; Miyajima et al., 2014).

1.3 Liver progenitor/stem cells

In adult organisms, liver turnover is achieved solely by the division of mature hepatocytes and biliary epithelial cells (Ponder, 1996). The robust proliferative ability of mature hepatocytes is also sufficient to recover the lost tissue mass after two-thirds partial hepatectomy. No involvement of liver progenitor/stem cells has been observed during either adult liver turnover or following two-thirds partial hepatectomy. However, when the proliferative ability of native hepatocytes and/or biliary epithelial cells is attenuated by exposure to chemicals such as dipin (Factor et al., 1994), diethoxycarbonyl-1, 4-dihydro-collidine (Preisegger et al., 1999), and diethylnitosamine (Schwarze et al., 1984), the resident liver progenitor/stem cell compartment becomes activated. Currently, this native stem cell population is called oval cells. Oval cells are small-sized cells having a high cytoplasm-to-nucleus ratio. As the name suggests, they can be identified by their characteristic oval shaped cell body. These cells are situated adjacent to the bile ducts and the canals of Hering. They are found to express a variety of markers, such as stem cell markers, hepatocyte markers, and biliary epithelial cell markers. However, the origins of these cells still remain unknown.

Recent studies have also shown the role of hepatic stellate cells (HSCs) as liver progenitor/stem cells (Yang et al., 2008). Hepatic stellate cells are star shaped cells located in the space of Disse. They make up 5-8% of the total cells in the liver. They perform various functions such as vitamin A storage (Gressner et al., 2006, 2008;

Friedman., 2008; Kisseleva et al., 2008), blood flow regulation (Wirz et al., 2008; Kisseleva et al., 2008) and produce several growth factors, such as hepatocyte growth factor (Tomiya et al., 2002; Schirmacher et al., 1992; Ramadori et al., 1992), insulin growth factor (Sanz et al., 2005), and fibroblast growth factor (Evarts et al., 1993). Hepatic stellate cells are shown to express many stem cell markers, such as c-kit (Fujio et al., 1994), nestin (Niki et al., 1999), p75 receptors (Trim et al., 2000), and CD133 (Kordes et al., 2007). The potential of these cells to differentiate into various cells of the liver has been demonstrated *in vitro* (Kordes et al., 2007).

1.4 Pregnancy-induced maternal liver growth

During pregnancy, in order to ensure the proper growth and development of the fetus, various maternal organs undergo structural and functional adaptations (Shingo et al., 2003; Nielsen et al., 1999; Huang et al., 2009; Kim et al., 2010; Audus, Soares, & Hunt, 2002; Dai et al., 2011; Rahman & Wendon, 2002). To cope with the increasing metabolic and nutritional demands during gestation, the maternal liver undergoes robust growth to increase its functional capacity (Dai et al., 2011). Our previous studies have shown that the maternal liver almost doubles in size by the end of pregnancy in rats and mice. This significant increase in maternal liver tissue mass is achieved by both compensatory hyperplasia and hypertrophy of hepatocytes (Bustamante et al., 2010; Dai et al., 2011; Milona et al., 2010). Compared to non-pregnant female mice, pregnant mice also showed changes in hepatic gene expression profiles (Bustamante et al., 2010).

1.5 *Ascl1*

The basic helix-loop-helix (bHLH) superfamily represents a group of proteins that functions as transcription factors in cells. Structurally, all bHLH proteins are comprised of two alpha helices which are joined together by a loop. One of the alpha helices contains a basic DNA binding region. ASCL1 (Achaete-scute homolog 1 protein) belongs to the basic helix-loop-helix (bHLH) family of transcription factors (Garcia-Bellido, 1979). In order to carry out its function the ASCL1 protein first dimerizes with another group of proteins called E-proteins. E-proteins are commonly expressed bHLH proteins such as Tcf4, E12, etc. They prime ASCL1 to bind efficiently to DNA (Murre et al., 1989; Bertrand, Castro, & Guillemot, 2002). Studies have shown that the *Ascl1* gene is required by neuronal progenitor cells to differentiate into neurons during embryonic neurogenesis (Bertrand, Castro, & Guillemot, 2002). ASCL1, along with Neurog1, signals neural progenitors to exit cell cycle by triggering the expression of a cyclin-dependent kinase inhibitor-p27 (Kip1) while simultaneously inducing expression of genes responsible for neuronal differentiation such as NeuroM (Farah et al., 2000; Fode et al., 2000). *Ascl1* plays a role in subtype specification and development of ventral neuroendocrine neurons (McNay et al., 2006). In mice, downregulation of *Ascl1* results in gliogenesis in the central nervous system (Tomita et al., 2000). ASCL1 suppresses the expression of genes such as Dlx1 and 2 to facilitate oligodendrogenesis in the cerebral cortex (Petriniak et al., 2007). *Ascl1* also promotes the differentiation of GABAergic neurons (Virolainen et al., 2012), olfactory receptor neurons (Cau et al., 1997), retinal neurons (Nelson et al., 2009; Hatakeyama et al., 2001; Tomita et al., 2000), pulmonary neuroendocrine cells (Borges et al., 1997), and gastric neuroendocrine cells (Kokubu et

al., 2008). In conclusion, *Ascl1* plays a critical role in the maintenance and differentiation of neuronal progenitor cells in the nervous system.

1.6 Hypothesis

We hypothesize that *Ascl1*-expressing cells are hepatic progenitor/stem cells that can commit to a hepatocyte lineage and that, during pregnancy, proliferate and differentiate into mature hepatocytes thereby repopulating a significant portion of the mouse maternal liver.

CHAPTER 2 MATERIALS AND METHODS

2.1 Animal care

Protocol for the care and use of animals was sanctioned by the Indiana University-Purdue University Indianapolis Animal Care and Use Committee. Animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals authorized by the National Institute of Health (NIH). Mice were housed in plastic cages and provided with food and water *ad libitum*. Temperature and relative humidity in the animal house were maintained at 22±1°C and 40-60%, respectively. Animals were placed on a 12-hr light:12-hr dark cycle with lights switched on from 06:00 hrs to 18:00 hrs. Colony size was expanded by breeding male and female mice having appropriate genotypes. Young mice were weaned, ear tagged, and genotyped using genomic DNA extracted from ear tissue samples.

2.2 Mouse models

2.2.1 Ascl1$^{GFP/+}$ reporter mice

The Ascl1^{tm1Reed}/J mice (hereafter referred to as Ascl1$^{GFP/+}$ mice), stock #012881, were purchased from Jackson Laboratory, Maine, USA (**Table 1**). The genotypes of mice

were identified by amplifying particular genes of interest using the polymerasechain reaction (PCR). PCR instructions were provided by the vendor. The master mix solution for the PCR was prepared using the Kapa Taq PCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA). All primers used were purchased from Integrated DNA Technologies (Coralville, IA, USA). The *Ascl1*wild type (wt) gene, coding for the ASCL1 protein, and its mutant $Ascl1^{GFP}$ allele, coding for the green fluorescent protein (GFP), were amplified. Primers 10841 and 10842 (**Table 2**) were used for the *Ascl1* wt allele. Primers 10843 and 10844 (**Table 2**) were used for the mutant allele. A 1.5% agarose gel with ethidium bromide was used to resolve the PCR products. DNA bands were visualized using a UV transilluminator. Band sizes of *Ascl1* wt allele and the $Ascl1^{GFP}$ mutant allele were 418bp and ~850bp respectively.

2.2.2 $Rosa26^{floxstopLacZ/floxstopLacZ}$;$Ascl1^{CreERT2/+}$ mouse line

The mouse line was generated by cross-breeding the following strains of mice mentioned below. Heterozygous F1 (first filial) generation was selfed to give rise to $Rosa26^{floxstopLacZ/floxstopLacZ}$;$Ascl1^{CreERT2/+}$ individuals.

The B6.129S4-*Gt(ROSA)26Sor*^{tm1Sor}/J mice (hereafter referred as $Rosa26^{floxstopLacZ}$ mice), stock #003474, were purchased from Jackson Laboratory, Maine, USA (**Table 1**). The genotypes of mice were identified by amplifying particular genes of interest using PCR. PCR instructions were provided by the vendor. The master mix solution for the PCR was prepared using the Kapa Taq PCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA). All primers used were purchased from Integrated DNA Technologies

(Coralville, IA, USA). The Rosa26 (RS26) wt gene and its mutant $RS26^{floxstopLacZ}$ allele, coding for the bacterial β-galactosidase enzyme, were amplified. Primers oIMR8545 and oIMR8546 (**Table 2**) were used for the RS26 wt allele. Primers oIMR8545 and oIMR8052 (**Table 2**) were used for the mutant allele. A 1.5% agarose gel with ethidium bromide was used to resolve the PCR products. DNA bands were visualized using a UV transilluminator. Band sizes of the RS26 wt allele and the $RS26^{floxstopLacZ}$ mutant allele were ~650bp and 340bp respectively.

The $\mathit{Ascl1}^{tm1.1(Cre/ERT2)Jejo}$/J mice (hereafter referred as $Ascl1^{CreERT2}$), stock #012882, were purchased from Jackson Laboratory, Maine, USA (**Table 1**). The genotypes of mice were identified by amplifying particular genes of interest using PCR. PCR was performed by modifying instructions provided by the vendor. The master mix solution for the PCR was prepared using the Kapa LongRange HotStart PCR kit (#KK3501, Kapa Biosystems, Inc., MA, USA). All primers used were purchased from Integrated DNA Technologies (Coralville, IA, USA). The *Ascl1* wt gene and its transgenic $Ascl1^{CreERT2}$ allele, coding for the mutant estrogen receptor fused with Cre recombinase enzyme, were amplified. Primers 10841 and 10842 (**Table 2**) were used for the *Ascl1* wt allele. Primers 10843 and 10653 (**Table 2**) were used for the transgenic allele. A 1.5% agarose gel with ethidium bromide was used to resolve the PCR products. DNA bands were visualized using a UV transilluminator. Band sizes of the *Ascl1* wt allele and the $Ascl1^{CreERT2}$ mutant allele were 418bp and ~300bp respectively.

2.2.3 GFAP-rtTA$^{tg/+}$;tetO-Cre$^{tg/+}$;Rosa26$^{floxstopEYFP/floxstopEYFP}$;Ascl1$^{flox/flox}$ mouse line

The mouse line was generated by cross-breeding the following strains of mice mentioned below in a strategic fashion, to give rise to GFAP-rtTA$^{tg/+}$;tetO-Cre$^{tg/+}$;Rosa26$^{floxstopEYFP/floxstopEYFP}$;Ascl1$^{flox/flox}$ individuals. Individuals with the genotype of GFAP-rtTA$^{tg/+}$;tetO-Cre$^{tg/+}$;Rosa26$^{floxstopEYFP/floxstopEYFP}$;Ascl1$^{+/+}$ were used as genetic controls.

The B6.Cg-Tg(GFAP-rtTA*M$_2$)1Rmra/J mice (hereafter referred to as GFAP-rtTA $^{tg/+}$ mice), stock #014098, were purchased from Jackson Laboratory, Maine, USA (**Table 1**). The genotypes of mice were identified by amplifying particular genes of interest using PCR. PCR instructions were provided by the vendor. The master mix solution for the PCR was prepared using the Kapa Taq PCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA). All primers used were purchased from Integrated DNA Technologies (Coralville, IA, USA). An internal positive control gene and the GFAP-rtTA^{tg} transgene, coding for the reverse tetracycline-controlled transactivator protein, were amplified. Primers oIMR7338 and oIMR7339 (**Table 2**) were used for the internal positive control gene. Primers 12933 and 12934 were used for the transgene (**Table 2**). A 1.5% agarose gel with ethidium bromide was used to resolve the PCR products. DNA bands were visualized using a UV transilluminator. Band sizes of the internal positive control gene and the GFAP-rtTA^{tg} transgene were 324bp and 200bp respectively.

The B6.Cg-Tg(tetO-cre)1Jaw/J mice (hereafter referred to as tetO-Cre$^{tg/+}$ mice), stock #006234, were purchased from Jackson Laboratory, Maine, USA (**Table 1**). The genotypes of mice were identified by amplifying particular genes of interest using PCR.

PCR was performed by modifying instructions provided by the vendor. The master mix solution for the PCR was prepared using the Kapa Taq PCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA). All primers used were purchased from Integrated DNA technologies (Coralville, IA, USA). An internal positive control gene and the tetO-Cre^{tg} transgene, coding for the Cre-recombinase enzyme, were amplified. Primers oIMR7338 and oIMR7339 (**Table 2**) were used for the internal positive control. Primers oIMR1084 and oIMR1085 (**Table 2**) were used for the transgene. A 1.5% agarose gel with ethidium bromide was used to resolve the PCR products. DNA bands were visualized using a UV transilluminator. Band sizes of the internal positive control gene and the tetO-Cre^{tg} transgene were 324bp and 100bp respectively.

The $Rosa26^{floxstopEYFP/floxstopEYFP}$;$Ascl1^{flox/flox}$ mice (**Table 1**) were donated to us by Dr. Guillemot (MRC National Institute for Medical Research, London). The genotypes of mice were identified by amplifying particular genes of interest using PCR. PCR protocols used were modified from Pacary et al., 2011. The master mix solutions for the PCRs were prepared using the Kapa Taq PCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA). All primers used were purchased from Integrated DNA Technologies (Coralville, IA, USA). Genes amplified were as follows: 1) RS26 wt allele; 2) $RS26^{floxstopEYFP}$; 3) *Ascl1* wt allele; and 4) $Ascl1^{flox}$. Primers RYFP-R1 and RYFP-R3 were used for the RS26 wt allele. Primers RYFP-R1 and RYFP-R2 were used for the $RS26^{floxstopEYFP}$. Primers MF1 and VR2 were used for the *Ascl1* wt allele. Primers MF1 and MR2 were used for the $Ascl1^{flox}$ gene. Primer details are mentioned in **Table 2**. A 1.5% agarose gel with ethidium bromide was used to resolve the PCR products. DNA bands were visualized

using a UV transilluminator. Band sizes of the genes were as follows: 1) RS26 wt allele-650bp; 2) RS26 ^{floxstopEYFP}-340bp; 3) *Ascl1* wt allele-~442bp; and 4) Ascl1^{flox}-857bp.

2.2.4 Rosa26$^{floxstopLacZ/floxstopLacZ}$;Ttr$^{CreERT2/+}$ mouse line

The mouse line was generated by cross-breeding the following strains of mice mentioned below. Heterozygous F1 (first filial) generation was selfed to give rise to Rosa26$^{floxstopLacZ/floxstopLacZ}$;Ttr$^{CreERT2/+}$ individuals.

The Tg(Ttr-cre/Esr1*)1Vco mice (hereafter referred to as Ttr^{CreERT2} mice) were purchased from Inserm, Paris, France (**Table 1**). The genotypes of mice were identified by amplifying particular genes of interest using PCR. Dr. Mireille Vasseur-Cognet provided us the PCR protocol. The master mix solution for the PCR was prepared using the Kapa Taq PCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA). All primers used were purchased from Integrated DNA technologies (Coralville, IA, USA). An internal positive control gene and the Ttr^{CreERT2} transgene, coding for the Cre-ERT2 fusion protein, were amplified. Primers Amorce USF int2 and Amorce USF int4 (**Table 2**) were used for the internal positive control gene. Primers Amorce Cre 26 and Amorce Cre 36 (**Table 2**) were used for the transgene. A 1.5% agarose gel with ethidium bromide was used to resolve the PCR products. DNA bands were visualized using a UV transilluminator. Band sizes of the internal positive control gene and the Ttr^{CreERT2} transgene were 600bp and 400bp respectively.

The Rosa26^{floxstopLacZ} mice are described are described in section 2.3.2.

2.3 Timed pregnancies

Appropriate numbers of breeding cages were setup at noon. Next day onwards, the vaginal opening of female mice were visually examined for a copulatory plug. Presence of a copulatory plug was considered as gestation day 1. Plug-positive female mice were separated from their male partners and housed individually.

2.4 Tamoxifen preparation and administration

Required amount of tamoxifen (#T5648-1G, Sigma-Aldrich) was dissolved in a vehicle solution of 10% ethanol and sesame oil (#S3547-1L, Sigma-Aldrich) to give a 24mg/ml working solution. Tamoxifen was dissolved thoroughly by incubating the working solution overnight on a shaker at 37°C. Pregnant mice were injected intraperitoneally with either tamoxifen (dissolved in vehicle) or vehicle only solutions on gestation days 10, 11, and 12. Pregnant mice were administered with a dose of 60mg/kg body of tamoxifen once each day between 10 to 11 am.

2.5 Tissue collection

Pregnant mice were sacrificed by cervical dislocation and total body weight, total maternal liver weight, and litter size of individual mouse was recorded. Maternal livers were frozen in optimal cutting temperature (OCT) medium (#4583 Tissue-Tek, Sakura)

cooled with heptane on dry ice. Ten micrometer thick sections of maternal livers were prepared from various gestation days and stored at -80ºC until further use.

2.6 Immunofluorescence staining

Frozen maternal liver sections from appropriate gestation days were taken out from -80ºC and allowed to dry at room temperature for 30 minutes. Using the liquid blocker PAP pen (Daido Sangyo Co. Ltd, Tokyo, Japan), boundaries were made around the tissue sections to contain the reagents over the tissues. Slides were then transferred into humidified chambers and fixed using ice cold 4% paraformaldehyde (#15735-10-S, Electron Microscopy Science) at 4ºC for 10 minutes. Slides were washed in 1X phosphate buffered saline (PBS) twice for 5 minutes each. Maternal livers were then quenched using 0.3% hydrogen peroxide solution for 10 minutes. Afterwards, maternal liver sections were blocked with 2% rabbit serum, which was diluted in 1X Dulbecco's phosphate buffered saline (DPBS) (#114-059-101, Quality Biological, Inc.) with Ca^{+} and Mg^{+}, for 1 hour at room temperature. Next, slides were incubated overnight with a primary antibody against GFP (#ab6673, Abcam). The antibody was diluted in 1X DPBS with Ca^{+} and Mg^{+} (1:100). The next day, slides were washed two times in 1X PBS for 5 minutes each. Tissues were then incubated with the rabbit anti-goat fluorescent secondary antibody (#305-516-046, Dylight™ 594, Jackson Immunoresearch Laboratories, Inc.) for 1 hour at room temperature. Tissues were washed in 1X PBS twice for 5 minutes each and mounted with ProLong Gold Antifade Mountant with DAPI (P36931, Life

Technologies). GFP immunolabeled maternal liver sections were visualized using the confocal microscope (Olympus FluoView FV1000).

2.7 LacZ staining

Frozen maternal liver sections from appropriate gestation days were taken from -80°C and allowed to dry at room temperature for 30 minutes. Using the liquid blocker PAP pen (Daido Sangyo Co. Ltd, Tokyo, Japan), boundaries were made around the tissue sections to contain the reagents that they were being incubated in. Tissue sections were fixed in freshly prepared LacZ fixative solution for 5 minutes. Slides were then washed twice in 1X PBS for 5 minutes each. Maternal liver sections were treated with LacZ staining solution for 12 hours. Sections were washed again in 1X PBS to clear the tissue of the LacZ staining solution. The LacZ fixative solution and the LacZ staining solution were prepared as per kit instructions (#rep-lz-t, InvivoGen). Following this, tissue sections were counterstained with hematoxylin (Hematoxylin 560 MX, Leica Biosystems) and mounted using the Vectamount™ AQ mounting medium (#H-5501, Vector Laboratories, Inc.).

2.8 LacZ and HNF4α co-staining

LacZ staining was first performed on frozen maternal liver sections from appropriate gestation days (refer to LacZ staining described in section 2.7). Tissues were washed twice in 1X PBS for 15 minutes each. Slides were then transferred into

humidified chambers and fixed using ice cold 4% paraformaldehyde at 4ºC for 10 minutes. Slides were washed in 1X PBS twice for 5 minutes each. Maternal livers were then quenched using 0.3% hydrogen peroxide solution for 10 minutes. After this, maternal liver sections were blocked with 2% donkey serum, which was diluted in 1X DPBS with Ca^{+} and Mg^{+}, for 1 hour at room temperature. Following this, slides were incubated overnight with a primary antibody against HNF4α (#sc-6556, Santa Cruz Biotechnology, Inc.). The antibody was diluted in 1X DPBS with Ca^{+} and Mg^{+} (1:100). The next day, slides were washed two times in 1X PBS for 5 minutes each. Tissues were then incubated with the biotinylated donkey anti-goat secondary antibody (#sc-2042, Santa Cruz Biotechnology, Inc.) for 1 hour at room temperature. Slides were then washed with 1X PBS two times for 5 minutes each and incubated for 30 minutes with the VECTASTAIN ABC kit reagent (Vector Laboratories, Inc., Burlingame, CA). Afterwards, maternal livers were treated with DAB Peroxidase Substrate Kit reagent (#SK-4100, Vector Laboratories, Inc.) and counterstained with hematoxylin. Excess hematoxylin was washed away using distilled water and tissues were mounted using the Vectamount™ AQ mounting medium. (#H-5501, Vector Laboratories, Inc.).

CHAPTER 3 RESULTS

3.1 Characterization of *Ascl1*-expressing cells during pregnancy using Ascl1$^{GFP/+}$ reporter mice

To test our hypothesis, the first aim was to identify *Ascl1*-expressing cells in the maternal liver during different days of gestation. We used the Ascl1$^{GFP/+}$ transgenic reporter mouse line. The Ascl1$^{GFP/+}$ mouse line was generated by deleting the coding region of the *Ascl1* gene and replacing it with a DNA sequence coding for the green fluorescent protein (GFP), which contains a nuclear localization sequence. The deletion was made on one allele of the *Ascl1* gene, while keeping the endogenous Ascl1 promoter intact, resulting in mice heterozygous for *Ascl1* (Ascl1$^{GFP/+}$). All cells with an active Ascl1 promoter therefore express GFP that is localized in the nucleus (**Figure 1**). Timed pregnancies were generated and maternal livers were collected on gestation days 11 (D11), 13 (D13), 15 (D15) and 18 (D18). Non-pregnant (NP) mice livers were used as control.

3.1.1 Maternal liver growth pattern in Ascl1$^{GFP/+}$ transgenic mice

Gravimetric measurements of total body weight and total liver weight were recorded from NP and pregnant D11, D13, D15, and D18 mice. We observed that during the second half of pregnancy the total liver weight almost doubled. The liver-to-body weight ratio increased significantly on D13 and D15 (**Figure 2**). These changes were indicative of robust maternal liver growth during pregnancy. The results were similar to that observed in our previous studies (Zou et al., 2013 and Dai et al., 2011). Thus, the genetic modification in Ascl1$^{GFP/+}$ mice does not affect maternal liver growth during pregnancy.

3.1.2 Preliminary identification of *Ascl1*-expressing cells in the maternal liver

NP and pregnant (D15) frozen maternal liver sections were immunolabeled with a GFP antibody and visualized using fluorescence microscopy. We found that *Ascl1*-expressing cells were observed in pregnant D15 maternal livers but not in NP livers. *Ascl1*-expressing cells were distributed randomly and uniformly throughout the liver parenchyma. Morphologically, *Ascl1*-expressing cells appeared as hepatocyte-like cells (**Figure 3**).

3.2 Lineage tracing of *Ascl1*-expressing cells in maternal liver during pregnancy

In order to trace the lineage of *Ascl1*-expressing cells in the maternal liver during pregnancy, we generated a mouse strain described in the following section.

3.2.1 Generation of $Rosa26^{floxstopLacZ/floxstopLacZ}$;$Ascl1^{CreERT2/+}$ mouse line

The mouse strain was generated by crossbreeding $Rosa26^{floxstopLacZ/ floxstopLacZ}$ mice with the $Ascl1^{CreERT2/+}$ mice.

$Rosa26^{floxstopLacZ/floxstopLacZ}$ mice: this mouse strain contains the gene for β-galactosidase (LacZ) enzyme, which is regulated by the Rosa26 (RS26) ubiquitous promoter. However, the expression of LacZ is prevented due to the presence of a flox stop cassette downstream of the RS26 promoter.

$Ascl1^{CreERT2/+}$ mice: this mouse strain contains the gene for the CreERT2 fusion protein. The *Ascl1* protein-coding sequence from one allele of the *Ascl1* gene was swapped with the CreERT2 coding sequence while preserving the endogenous Ascl1 promoter. CreERT2 fusion protein is a combination of Cre-recombinase enzyme fused to a mutant form of the human estrogen receptor. This mutant form of the human estrogen receptor is designed to be unable to bind to its natural ligand, estrogen. Instead, it binds to tamoxifen, a synthetic estrogen receptor ligand molecule. Activation of the Ascl1 promoter, thus, leads to the expression of the CreERT2 fusion protein, which, in its inactive state, localizes in the cytoplasm of the cell. When activated by tamoxifen it translocates to the nucleus.

In the Rosa26$^{floxstopLacZ/floxstopLacZ}$;Ascl1$^{CreERT2/+}$ lineage tracing mouse model, production of Cre-recombinase enzyme is driven by the Ascl1 promoter while the RS26 promoter drives the production of LacZ enzyme. In the absence of tamoxifen, the LacZ transgene remains unexpressed due to the flox stop cassette. When tamoxifen is injected into the mouse, the activated CreERT2 fusion protein is translocated into the nucleus where the Cre-recombinase floxes out the flox stop sequence. This results in the production of LacZ enzyme in *Ascl1*-expressing cells (**Figure 4**). Therefore, LacZ permanently labels *Ascl1*-expressing cells and can be visualized by using X-Gal substrate.

3.2.2 Standardization of tamoxifen dose to induce LacZ expression in *Ascl1*-expressing cells

In order to induce the expression of LacZ enzyme in *Ascl1*-expressing cells in the maternal liver without compromising pregnancy, it was important to determine the optimal dose for tamoxifen. Timed pregnancies were generated and tamoxifen, dissolved in sesame oil containing 10% ethanol (vehicle), or vehicle only was administered either once or multiple times between gestation days 9 to 12. Gestation day 18 (D18) maternal livers were collected and analysed using LacZ staining protocol. We observed that out of the three doses of tamoxifen that were tested (30, 45 and 60 mg/kg body weight), 60 mg tamoxifen/kg body weight, when injected on gestation days 10, 11, and 12 (intraperitoneally, once each day), induced robust expression of LacZ enzyme in the maternal liver. The other two doses had no effect (**Figure 5**). Sixty mg/kg dose of

tamoxifen however did cause a small percent of abortion in mice. Nonetheless, we used this dose for *in vivo* lineage tracing studies. Additionally, we also observed that the volume of sesame oil injected into mice critically affected the outcome of pregnancy. Pregnant mice injected with 150 µl of sesame oil (per mouse) caused abortions whereas mice injected with 75 µl (per mouse) of sesame oil showed no abortion. Thus, 75 µl (per 30 gm body weight) of sesame oil was set as the maximum vehicle volume that was injected in pregnant mice. We also tested the efficacy of 4-Hydroxytamoxifen (4-OHT), an active metabolite of tamoxifen (Reed et al., 2005). We found that 4-OHT at a concentration of 50 mg/kg body weight, when injected on gestation days 10, 11 and 12 (intraperitoneally, once each day), was unable to induce LacZ expression in D18 maternal livers (**Figure 6**).

Thus, a dose of 60mg/kg body weight of tamoxifen was used henceforth to induce the robust expression of LacZ in maternal hepatic *Ascl1*-expressing cells during pregnancy.

3.2.3 Lineage tracing of *Ascl1*-expressing cells

To determine whether *Ascl1*-expressing cells belong to a population of hepatic progenitor/stem cells, which differentiate into mature hepatocytes during the course of gestation, we performed the lineage tracing experiment in pregnant $Rosa26^{floxstopLacZ/floxstopLacZ}$;$Ascl1^{CreERT2/+}$ mice. Timed pregnancies were generated and tamoxifen (60 mg/kg body weight), dissolved in vehicle, or vehicle alone was administered intraperitoneally once each day on gestation days 10, 11 and 12. Gestation

day 13 (D13) and 18 (D18) maternal livers were collected and frozen in OCT cooled with heptane on dry ice. Ten micrometer thick frozen maternal liver (D13 and D18) sections were prepared and analysed using the LacZ staining protocol. LacZ labeled cells were seen in both D13 and D18 maternal livers. These LacZ-expressing cells morphologically resembled hepatocytes. There was a drastic increase in the number of LacZ-expressing cells from D13 to D18, which were spread throughout the liver parenchyma and the periportal regions. On D13, the LacZ-expressing cells were found in small patches across the maternal liver. On D18, these cells constituted almost the entire maternal liver (**Figure 7**). Following this, to determine the identity of the LacZ-expressing cells, D13 and D18 maternal liver sections were subjected to LacZ and HNF4α co-staining. Results showed the presence of two distinct populations of LacZ-expressing cells in the maternal liver, both on D13 and D18. One population was $LacZ^+$, $HNF4\alpha^+$ and the other was $LacZ^+$, $HNF4\alpha^-$. On D13, both populations were small and roughly equal in number. Compared to D13, on D18 the two populations were greater in number. Additionally, on D18, maternal livers had a greater number of $LacZ^+$, $HNF4\alpha^+$ cells compared to $LacZ^+$, $HNF4\alpha^-$ cells. We concluded that during the course of gestation, *Ascl1*-expressing cells differentiate along a hepatocyte lineage by undergoing asymmetric stem cell division, thereby repopulating the maternal liver.

3.3 Generation of hepatic stellate cell-specific *Ascl1* conditional knockout (cKO) mouse line

Previous studies have shown the ability of hepatic stellate cells (HSCs) to act as hepatic progenitor/stem cells during liver injury. Unpublished data from our lab showed ASCL1 expression in LX-2 cells (human hepatic stellate cell line) in culture. Therefore, we hypothesize that, *Ascl1* plays a crucial role in the transdifferentiation of HSCs into mature hepatocytes during pregnancy. In order to test our hypothesis we generated the GFAP-rtTA$^{tg/+}$;tetO-Cre$^{tg/+}$;Rosa26$^{floxstopEYFP/floxstopEYFP}$;Ascl1$^{flox/flox}$ cKO mouse model. These mice were generated by the sequential crossbreeding of the following mouse strains.

GFAP-rtTA$^{tg/+}$ mice: this mouse strain contains the GFAP-rtTA non-allelic transgene that is randomly inserted into the mouse genome. The gene contains an upstream glial fibrillary acidic protein (GFAP) promoter and a downstream protein-coding region that translates to reverse tetracycline-controlled transactivator protein (rtTA). Therefore, mice carrying the GFAP-rtTA transgene produce rtTA in all cells with an active GFAP promoter. The rtTA protein localizes in the cell cytoplasm and is activated by doxycycline (Dox). Activated rtTA translocates into the nucleus.
tetO-Cre$^{tg/+}$ mice: this mouse strain contains the tetO-Cre non-allelic transgene inserted randomly into the mouse genome. The transgene contains an upstream tetO promoter followed downstream by the region coding for the Cre-recombinase enzyme. The tetO promoter is switched on when active rtTA binds to the promoter element. This in turn results in expression of Cre-recombinase.

Rosa26$^{floxstopEYFP/floxstopEYFP}$;Ascl1$^{flox/flox}$ mice: this is a bi-transgenic mouse strain. Both transgenes are allelic. The RS26^{floxstopEYFP} transgene comprises the RS26 ubiquitous promoter that drives the expression EYFP. However, because of a floxed stop cassette present after the Rosa26 promoter, EYFP expression is stalled. The Ascl1$^{flox/flox}$ transgene comprises the Ascl1 promoter and the downstream *Ascl1* protein-coding region, which is flanked by a pair of loxP sites.

In the GFAP-rtTA$^{tg/+}$;tetO-Cre$^{tg/+}$;Rosa26$^{floxstopEYFP/floxstopEYFP}$;Ascl1$^{flox/flox}$ cKO mouse model, the GFAP promoter drives the expression of rtTA. When mice are exposed to Dox, rtTA is activated, translocates to the nucleus, binds to and activates the tetO promoter, which in turn drives the production of Cre-recombinase enzyme. Cre-recombinase floxes out the *Ascl1* gene and the stop cassette. The *Ascl1* gene is thereby conditionally knocked out from all GFAP positive hepatic stellate cells. In addition, these cells lacking the *Ascl1* gene are simultaneously and permanently labeled for EYFP (**Figure 9**). Mice having the genotype GFAP-rtTA$^{tg/+}$;tetO-Cre$^{tg/+}$;Rosa26$^{floxstopEYFP/floxstopEYFP}$;Ascl1$^{+/+}$ were used as genetic controls. Currently we are expanding the colony size to generate a sufficient number of mice for experimentation.

3.4 Generation of the hepatocyte fate tracing mouse line

Based on the data generated so far we believe that a significant portion of the maternal liver is repopulated by hepatocytes that are differentiated from hepatic progenitor/stem cells. The question then arises-what is the fate of the pre-existing

hepatocytes in the maternal liver during pregnancy? To answer this question, we are presently generating the Rosa26$^{floxstopLacZ/floxstopLacZ}$;Ttr$^{CreERT2/+}$ mouse model (**Figure 10**) to trace the fate of pre-existing hepatocytes. To generate this mouse model we crossbred the following mouse strains.

Rosa26$^{floxstopLacZ/floxstopLacZ}$ mice: this mouse strain is described in section 3.2.1 Ttr$^{CreERT2/+}$ mice: this mouse strain contains the transgene that codes for the CreERT2 fusion protein. The protein-coding region of one allele of the transthyretin (Ttr) gene is substituted by the CreERT2 protein-coding sequence. Therefore, mice expressing the Ttr^{CreERT2} transgene are thus heterozygous for Ttr. Ttr is a protein that is specifically expressed in mature hepatocytes. Hence, all hepatocytes containing this transgene express CreERT2 that is localized in the cytoplasm.

Thus, in the Rosa26$^{floxstopLacZ/floxstopLacZ}$;Ttr$^{CreERT2/+}$ mouse model, expression of CreERT2 fusion protein is driven by the Ttr promoter. This promoter is constantly active in mature hepatocytes. When exposed to tamoxifen, nuclear translocation of the activated CreERT2 fusion protein occurs. The Cre-recombinase enzyme then floxes out the stop cassette present in the RS26^{floxstopLacZ} transgene, thus permanently labeling mature hepatocytes with the LacZ enzyme.

Presently, we are in the process of generating mice that are homozygous for the RS26^{floxstopLacZ} gene and heterozygous for the Ttr^{CreERT2}.

CHAPTER 4 DISCUSSION

4.1 *Ascl1* expression in the maternal liver during pregnancy

Our current study shows, for the first time, the expression of *Ascl1* in the adult liver of mice, specifically, in the maternal liver during pregnancy. The phenomenon of robust maternal liver growth during pregnancy in CD-1 mice, C57BL/6 mice and rats has been reported previously by our group (Zou et al., 2013; Dai et al., 2011; Bustamante et al., 2010). In our current study, the maternal liver of the Ascl1$^{GFP/+}$ mouse strain exhibited the same response to pregnancy. Thus, the genetic manipulation of the Ascl1$^{GFP/+}$ mouse line does not affect the progression of pregnancy-induced maternal liver growth. The increase in liver weight is accompanied by the simultaneous increase in *Ascl1* mRNA expression from gestation day 8 to 18. The maximum fold change reached 2,500 fold on gestation day 15 (D15) relative to NP state (unpublished data from our laboratory). In a gene microarray analysis, among all the genes up-regulated during pregnancy, the *Ascl1* gene showed the highest fold change at mRNA level (unpublished data from our laboratory). The results of histological analysis of *Ascl1*-expressing cells, using the Ascl1$^{GFP/+}$ reporter mouse line, are consistent with the D15 *Ascl1* mRNA expression data. We observed the presence of GFP positive *Ascl1*-expressing cells on D15 (**Figure 3**). These *Ascl1*-expressing cells are randomly distributed throughout the maternal liver

parenchyma and morphologically appear as hepatocyte-like cells. To identify the *Ascl1*-expressing cell type in the maternal liver during pregnancy, co-immunolabeling studies with various cell-specific markers needs to be done.

Literature study on *Ascl1* so far has shown that *Ascl1* is associated with the development of cells of the nervous system, especially during embryonic neurogenesis (Kim et al., 2007). It is known that *Ascl1* is expressed by neuronal progenitors, such as neuroblasts of the central, peripheral and sympathetic nervous systems (Ma et al., 1996; Horton et al., 1999; Guillemot and Joyner., 1993; Gordon et al., 1996; Blaugrund et al., 1996 and Morikawa et al., 2009) and neural progenitors of olfactory receptor and retinal neurons (Krolewski et al., 2012; Nelson et al., 2009 and Hatakeyama et al., 2001). *Ascl1* is also expressed by differentiated cells of the nervous system, such as GABAergic neurons (Virolainen et al., 2012). In adult mice, the pituitary gland is shown to express ASCL1 (www.BioGPS.org). Here we have identified the liver as an adult organ highly expressing *Ascl1* during pregnancy.

4.2 Maternal liver repopulation by *Ascl1*-expressing cells

To determine the fate of *Ascl1*-expressing cells in the maternal liver during pregnancy, we generated the $Rosa26^{floxstopLacZ/floxstopLacZ}$;$Ascl1^{CreERT2/+}$ lineage tracing mouse model. *Ascl1*-expressing cells were labeled by LacZ enzyme between gestation days 10 to12. We found that LacZ positive *Ascl1*-expressing cells were present in the pregnant maternal livers. These LacZ positive cells were distributed in patches throughout the maternal liver parenchyma and around the periportal regions. Also,

compared to gestation day 13 (D13), the number of LacZ positive cells increased significantly on gestation day 18 (D18), contributing to more than half the maternal liver mass. Additionally, when LacZ-expressing cells were co-labeled with HNF4α, we found two distinct populations of LacZ-expressing cells: HNF4α^{+} and HNF4α^{-}. These two populations were seen in both D13 and D18 maternal livers. HNF4α^{+} LacZ expressing cells represent hepatocytes that are derived from *Ascl1*-expressing cells. These observations indicate that *Ascl1*-expressing cells undergo hyperplasia and differentiate into hepatocytes thereby repopulating the maternal liver during pregnancy. This is the most exciting finding of the current study. The finding implies that pregnancy induces the turnover of the maternal liver and also possibly other organs as well.

Many studies have shown evidence for the existence of hepatic progenitor/stem cells. Liver Progenitor Cells (LPC's) located in the canals of hering are considered as liver stem cells. Current theories suggest that during liver regeneration, biliary epithelial cells act as facultative stem cells. These cells give rise to LPC's in the periportal regions, which further differentiate into hepatocytes (Miyagima et al., 2014; Michalopoulos, 2013). LacZ labeled cells are mostly localized around the periportal regions of the maternal liver, suggesting that new hepatocytes may be differentiated from biliary epithelial cells. Co-immunolabeling of LacZ positive cells with LPC markers and mature hepatocyte markers needs to be done to determine the identity of the LacZ labeled cells. Moreover, we need to identify the *Ascl1*-expressing cells in the maternal liver prior to gestation day 13. We predict that the *Ascl1*-expressing cells are a population of liver progenitor cells. By gestation day 13, these *Ascl1*-expressing cells might have

differentiated into the hepatocyte lineage. That may explain why the LacZ-expressing cells are hepatocyte-like cells at gestation day 13 and thereafter.

Reports have implicated the ability of hepatic stellate cells (HSCs) to behave as liver progenitor cells. HSCs exhibit protein markers, namely Hes1, Desmin and GFAP that are characteristic of the three germ layers, the endoderm, mesoderm and ectoderm, respectively (Buchholz et al., 2005; Yokoi et al., 1984; Geerts et al., 1998; Neubauer et al., 1996; Buniatian et al., 1996). *In vitro* studies have described that, depending on conditions of the culture media, HSCs can differentiate into cells closely resembling hepatocytes, cells of the bile duct and endothelial cells (Kordes et al., 2007; Sicklick et al., 2006). HSCs also synthesize morphogens such as epimorphin, hepatocyte growth factor and pleitrophin (Yoshino et al., 2006; Hu et al., 1993; Asahina et al., 2002) and express stem cell markers such as CD133 and c-kit (Kordes et al., 2007; Fujio et al., 1994). A recent study, using the *in vivo* lineage tracing approach, also conclusively demonstrated the transdifferentiation of HSCs into mature hepatocytes. This study was carried out in transgenic mice that were fed with diets deficient in methionine choline and supplemented with ethionine to induce chronic liver injury. The fate of HSCs labeled with GFP was traced upon chemical induced chronic liver injury. Co-immunolabeling studies proved that GFP-positive HSCs differentiated into GFP-positive hepatocytes expressing albumin. These GFP-positive hepatocytes repopulated the injured liver after a period of one week post injury (Yang et al., 2008). We also found that *Ascl1* is expressed by the LX-2 human hepatic stellate cell line in culture (unpublished data from our laboratory). Thus, our hypothesis is that *Ascl1* regulates the ability of HSCs to differentiate into hepatocytes during pregnancy. To test this hypothesis we generated the

GFAP-rtTA$^{tg/+}$;tetO-Cre$^{tg/+}$;Rosa26$^{floxstopEYFP/floxstopEYFP}$;Ascl1$^{flox/flox}$ cKO mouse model. Using this mouse model, we could conditionally delete the *Ascl1* gene specifically from HSCs and trace the fate of these cells during the course of gestation. We also generated the GFAP-rtTA$^{tg/+}$;tetO-Cre$^{tg/+}$;Rosa26$^{floxstopEYFP/floxstopEYFP}$;Ascl1$^{+/+}$ reporter mouse model. Using this mouse line, we could determine the fate of HSCs without ablation of the *Ascl1* gene.

In summary, our study has shown that *Ascl1* expression is increased in the maternal liver during the second half of pregnancy. Morphologically, *Ascl1*-expressing cells look like hepatocytes. These *Ascl1*-expressing cells are distributed uniformly across the maternal liver parenchyma and the periportal regions. During the course of gestation, *Ascl1*-expressing cells proliferate and eventually repopulate a large portion of the maternal liver towards the end of pregnancy. However, the origin and identity of the *Ascl1*-expressing cells need to be determined. Our studies strongly suggest a critical role for *Ascl1* in maternal hepatic adaptations to pregnancy.

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TABLES

Table 1. List of mouse strains

Sr No.	Mouse Strain	Type	Mutant Gene	Promoter	Purpose
1.	Ascl1$^{GFP/+}$	Allelic	Ascl1-GFP	Ascl1	For identifying Ascl1-expressing cells in pregnant maternal livers
2.	Rosa26$^{floxstopLacZ/floxstopLacZ}$	Allelic	Rosa26-floxstopLacZ	Rosa26	For labeling specific cell types expressing Cre-recombinase with LacZ
3.	Ascl1$^{CreERT2/+}$	Allelic	Ascl1-CreERT2	Ascl1	For tracing the lineage of Ascl1-expressing cells in pregnant maternal livers
4.	GFAP-rtTA$^{tg/+}$	Transgene	GFAP-rtTA	GFAP	For tracing the lineage of hepatic stellate cell in pregnant maternal livers
5.	tetO-Cre$^{tg/+}$	Transgene	tetO-Cre	tetO	For tracing the lineage of hepatic stellate cells in pregnant maternal livers
6.	Rosa26$^{floxstopEYFP/floxstopEYFP}$;Ascl1$^{flox/flox}$	Allelic	1) Rosa26-floxstopEYFP 2) Ascl1-flox	1) Rosa26 2) Ascl1	For tracing the lineage of hepatic stellate cell in pregnant maternal livers
7.	Ttr$^{CreERT2/+}$	Allelic	Ttr-CreERT2	Ttr	For tracking the fate of pre-existing hepatocytes in pregnant maternal livers

Table 2: List of primers used for polymerase chain reaction

Sr No.	Primer Name	Primer Type	Primer Sequence 5’------>3’
1	10841	Forward	TCCAACGACTTGAACTCTATGG
2	10842	Reverse	CCAGGACTCAATACGCAGGG
3	10843	Forward	AACTTTCCTCCGGGGCTCGTTTC
4	10844	Reverse	TGGCTGTTGTAGTTGTACTCCAGC
5	oIMR8052	Reverse	GCGAAGAGTTTGTCCTCAACC
6	oIMR8545	Forward	AAAGTCGCTCTGAGTTGTTAT
7	oIMR8546	Reverse	GGAGCGGGAGAAATGGATATG
8	10653	Reverse	CGCCTGGCGATCCCTGAACATG
9	oIMR7338	Forward	CTAGGCCACAGAATTGAAAGATCT
10	oIMR7339	Reverse	GTAGGTGGAAATTCTAGCATCATCC
11	12933	Forward	GAAGGCGAGTCATGGCAAG
12	12934	Reverse	CAATACGCAGCCCAGTGTAAA
13	oIMR1084	Forward	GCGGTCTGGCAGTAAAAACTATC
14	oIMR1085	Reverse	GTGAAACAGCATTGCTGTCACTT
15	RYFP-R1	Forward	AAAGTCGCTCTGAGTTGTTAT
16	RYFP-R2	Reverse	AAGACCGCGAAGAGTTTGTC
17	RYFP-R3	Reverse	GGAGCGGGAGAAATGGATATG
18	MF1	Forward	CTACTGTCCAAACGCAAAGTGG
19	MR1	Reverse	TAGACGTTGTGGCTGTTGTAGT
20	VR2	Reverse	GCTCCCACAATCCTCGTAAAGA
21	Amorce USF int2	Forward	CCACCATGAGCCAGCAGTAATAC
22	Amorce USF int4	Reverse	TTGAAGCGTCCAATTATCACCC
23	Amorce Cre 26	Forward	CCTGCAAAATGCTTCTGTCCG
24	Amorce Cre 36	Reverse	CAGGCTATAAGCAATCCC

FIGURES

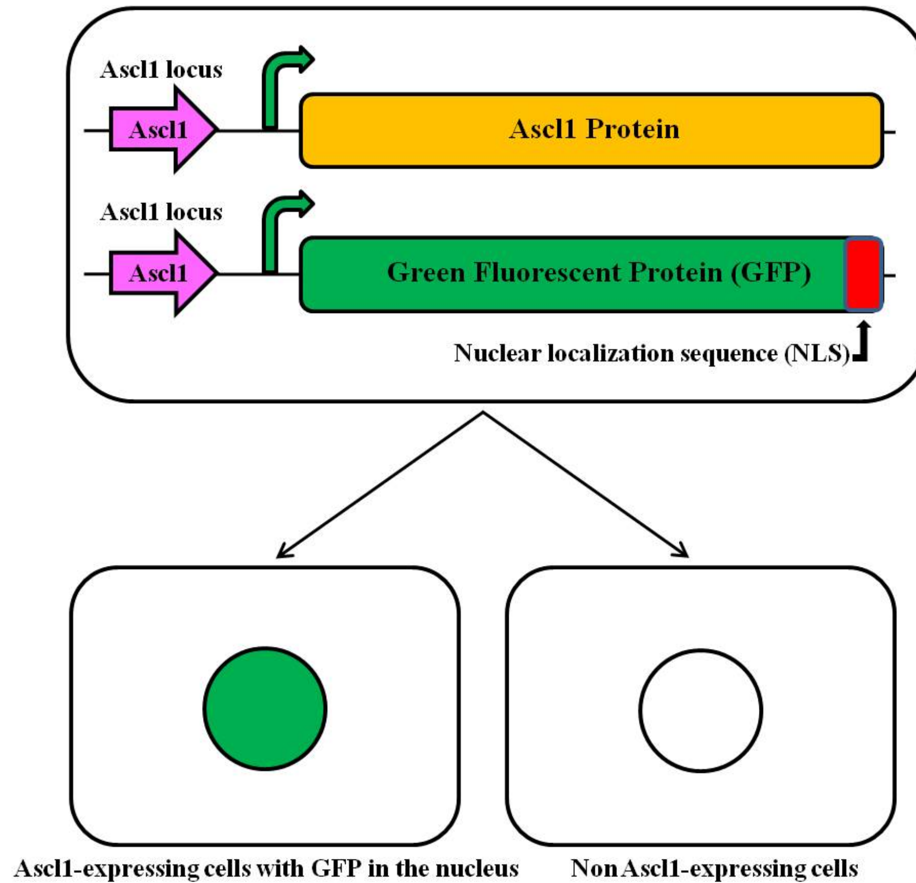

Figure 1: Genotype of Ascl1$^{GFP/+}$ reporter mouse line. In the Ascl1$^{GFP/+}$ mouse line, the *Ascl1* protein-coding region of one of the *Ascl1* alleles is substituted with the DNA sequence coding for GFP. This results in mice heterozygous for the *Ascl1* gene. Cells with an active Ascl1 promoter therefore express GFP, which localizes into the nuclei of *Ascl1*-expressing cells due to the presence of a nuclear localization sequence. Non-*Ascl1*-expressing cells do not express GFP.

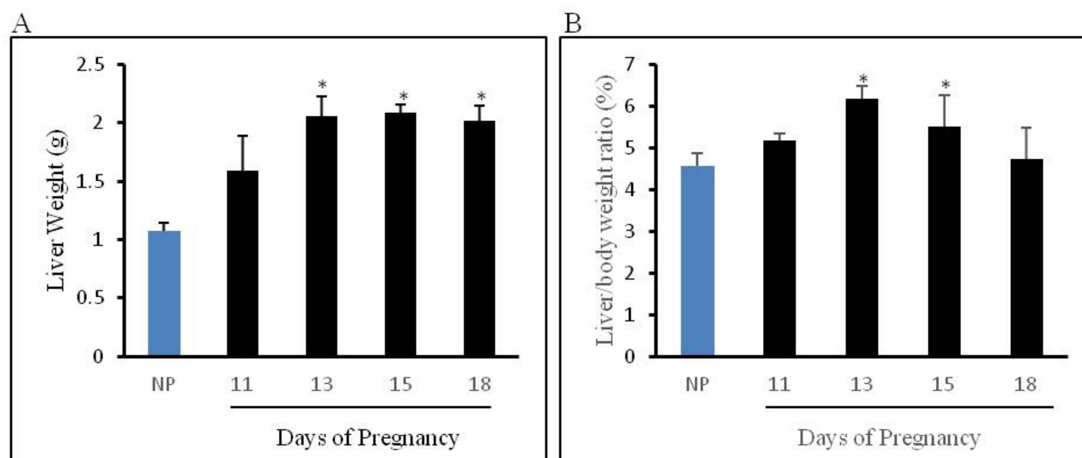

Figure 2: Response of maternal liver weight and liver-to-body weight ratios to pregnancy. Gravimetric measurements of total liver weights (**A**) and liver-to-body weight ratios (**B**) during pregnancy. Livers were collected from non-pregnant (NP) and pregnant days 2, 11, 13, 15 and 18 mice. Data are expressed as mean ± SEM (n=4). Asterisks indicate $P < 0.05$ in comparison with NP mice.

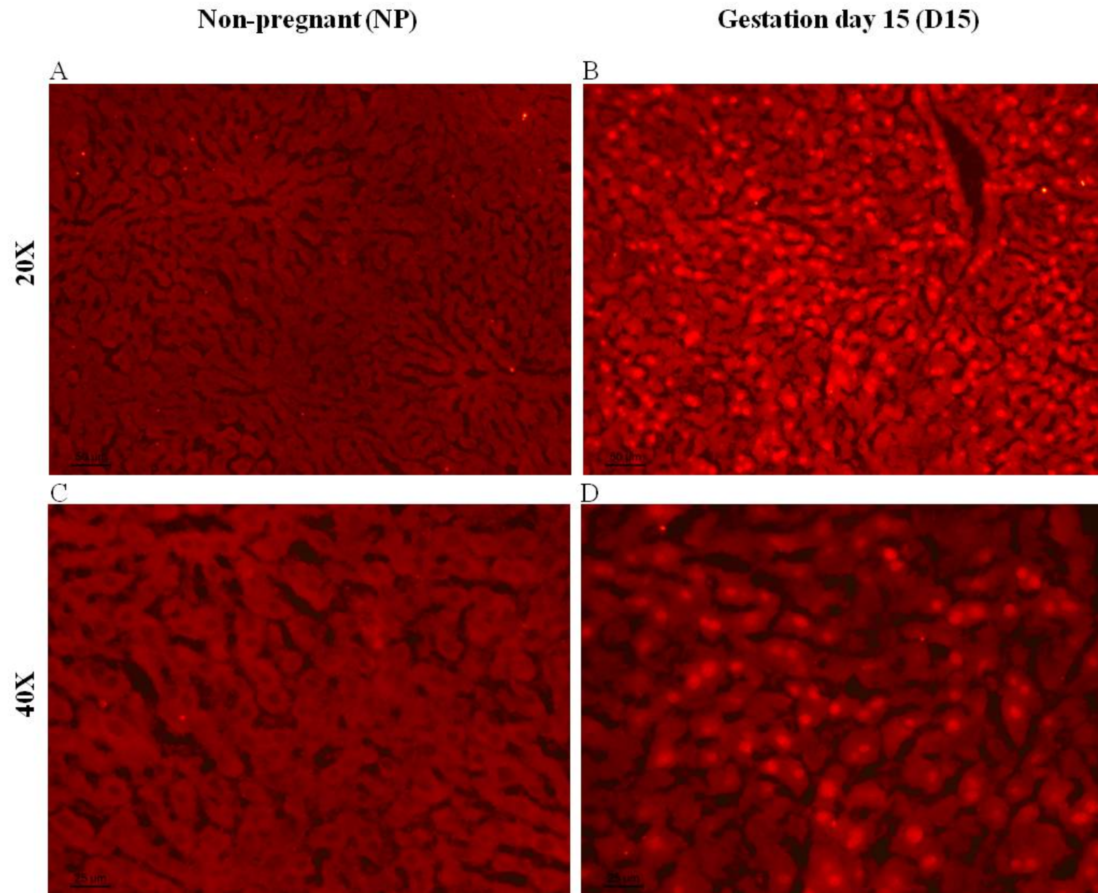

Figure 3: Immunofluorescence staining for green fluorescent protein (GFP). Non-pregnant and gestation day 15 (D15) maternal livers were harvested and frozen in OCT cooled with heptane on dry ice. Ten micrometer thick maternal liver sections were prepared and immunolabeled for GFP. NP and D15 maternal liver sections at 20X (**A** and **B**) and 40X (**C** and **D**) magnification. Hepatic *Ascl1*-expressing cells, while absent in NP maternal livers, were abundant in D15 maternal livers. *Ascl1*-expressing cells were randomly scattered throughout the liver parenchyma and around the periportal regions. Morphologically, *Ascl1*-expressing cells appeared as hepatocyte-like cells. Note: GFP-positive, *Ascl1*-expressing cells have red coloured nuclei.

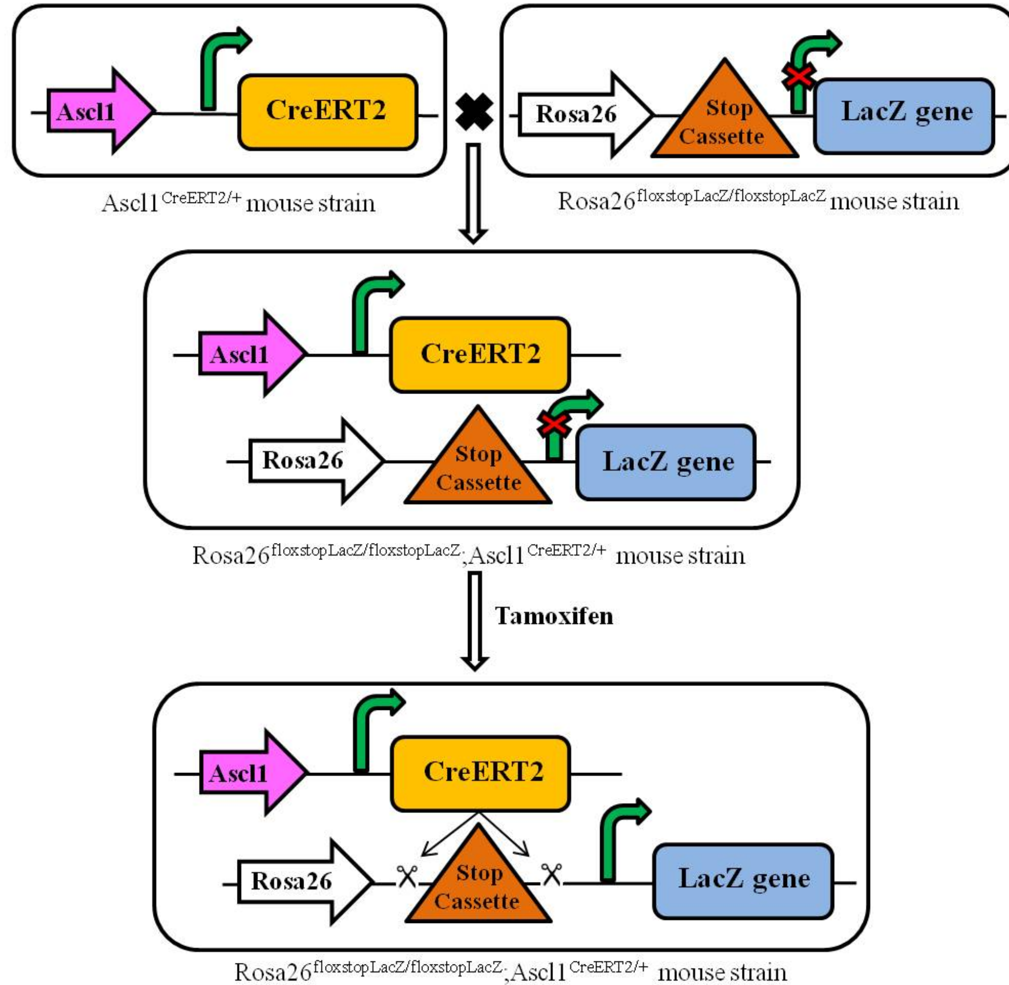

Figure 4: Generation of Rosa26$^{floxstopLacZ/floxstopLacZ}$;Ascl1$^{CreERT2/+}$ mouse model. This mouse model was generated by mating Rosa26$^{floxstopLacZ/floxstopLacZ}$ mice with Ascl1$^{CreERT2/+}$ mice. When the Rosa26$^{floxstopLacZ/floxstopLacZ}$;Ascl1$^{CreERT2/+}$ mice are treated with tamoxifen the CreERT2 fusion protein, expressed in *Ascl1*-expressing cells, is translocated into the nucleus. Inside the nucleus, the Cre recombinase enzyme floxes the stop cassette of the Rosa26^{floxstopLacZ} transgene. As a result, all *Ascl1*-expressing cells are perpetually labeled with LacZ enzyme.

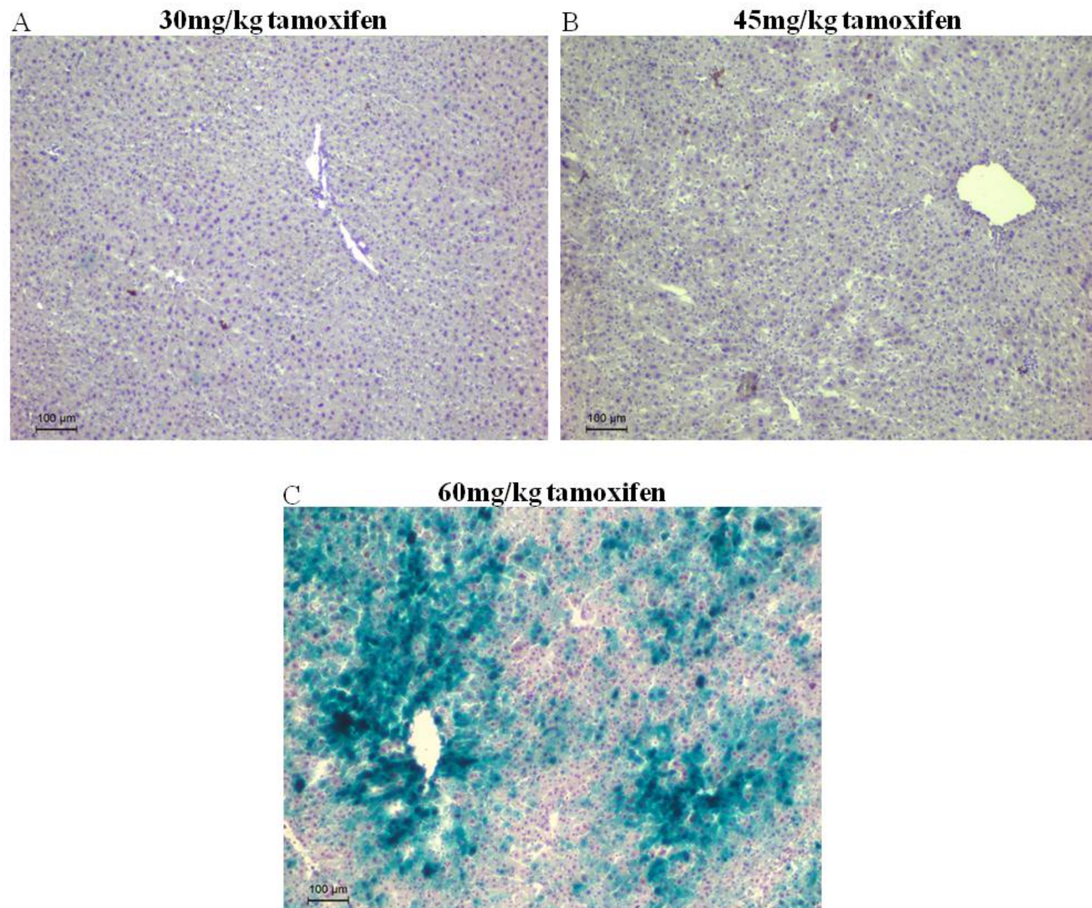

Figure 5: Standardization of tamoxifen dose to induce LacZ expression. Pregnant mice were injected with different doses of tamoxifen (30mg, 45mg, and 60mg/kg body weight) on gestation days 10, 11, and 12 (intraperitoneal, once each day). Gestation day 18 (D18) maternal livers were frozen in OCT cooled with heptane on dry ice. Ten micrometer thick maternal liver sections were prepared and stained for LacZ activity. While 30mg and 45mg (**A** and **B** respectively) of tamoxifen/kg body weight failed to induce LacZ expression, a dose of 60mg/kg induced robust LacZ expression in the maternal livers (**C**). Note: LacZ-expressing cells are blue in colour.

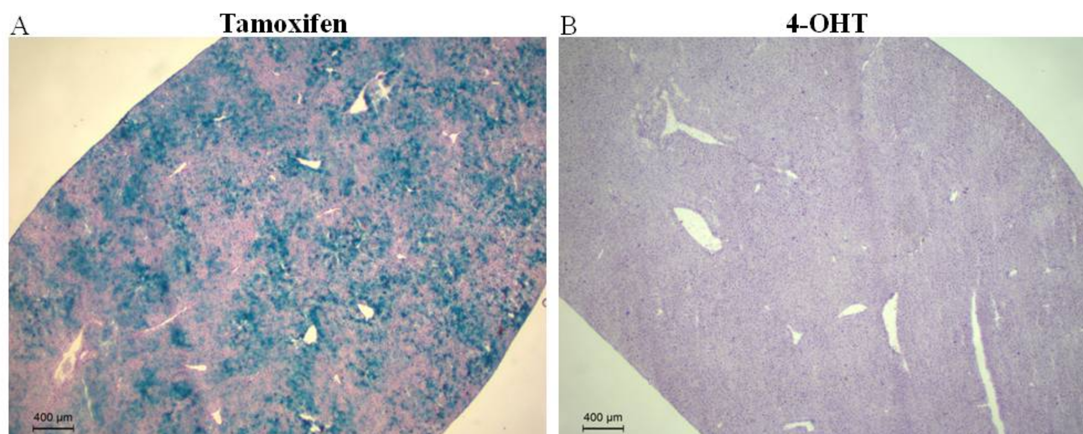

Figure 6: Efficacy of 4-Hydroxytamoxifen vs tamoxifen to induce LacZ expression. Pregnant mice were injected with 4-Hydroxytamoxifen (4-OHT) or tamoxifen on gestation days 10, 11, and 12 (intraperitoneal, once each day). Gestation day 18 (D18) maternal livers were harvested and frozen in OCT cooled with heptane on dry ice. Ten micrometer thick maternal liver sections were prepared and stained for LacZ activity. Compared to tamoxifen (**A**), 4-OHT (**B**) failed to induce expression of LacZ in pregnant maternal livers. Note: LacZ-expressing cells are blue in colour.

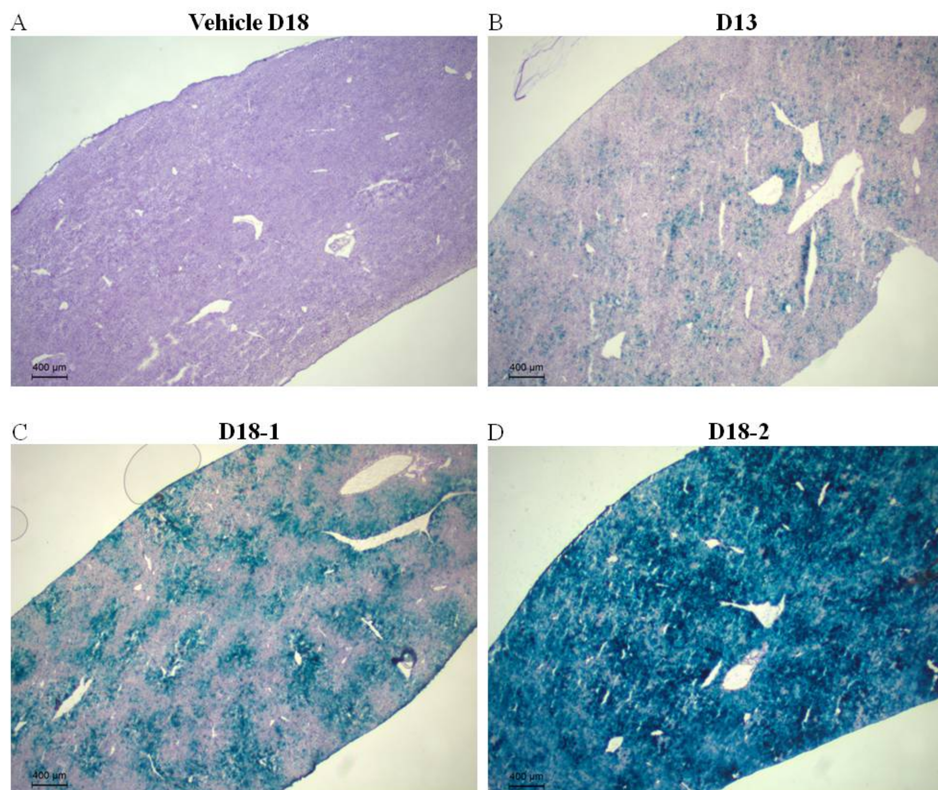

Figure 7: LacZ staining of maternal livers during pregnancy. Pregnant mice were injected with vehicle or tamoxifen (dissolved in vehicle) on gestation days 10, 11, and 12 (intraperitoneal, once each day). Gestation day 13 (D13) and 18 (D18) maternal livers were frozen in OCT cooled with heptane on dry ice. Ten micrometer thick maternal liver sections were prepared and stained for LacZ activity. (**A**) D18 maternal livers of mice injected with vehicle only. (**B**) D13 and (**C**-**D**) D18 maternal livers injected with tamoxifen. LacZ-expressing cells are distributed uniformly in the liver parenchyma and around portal triad and central vein. D13 LacZ-expressing cells undergo hyperplasia to repopulate the maternal liver as seen on D18. LacZ-expressing cells were found in the maternal liver parenchyma and around periportal regions. Note: LacZ-expressing cells are blue in colour.

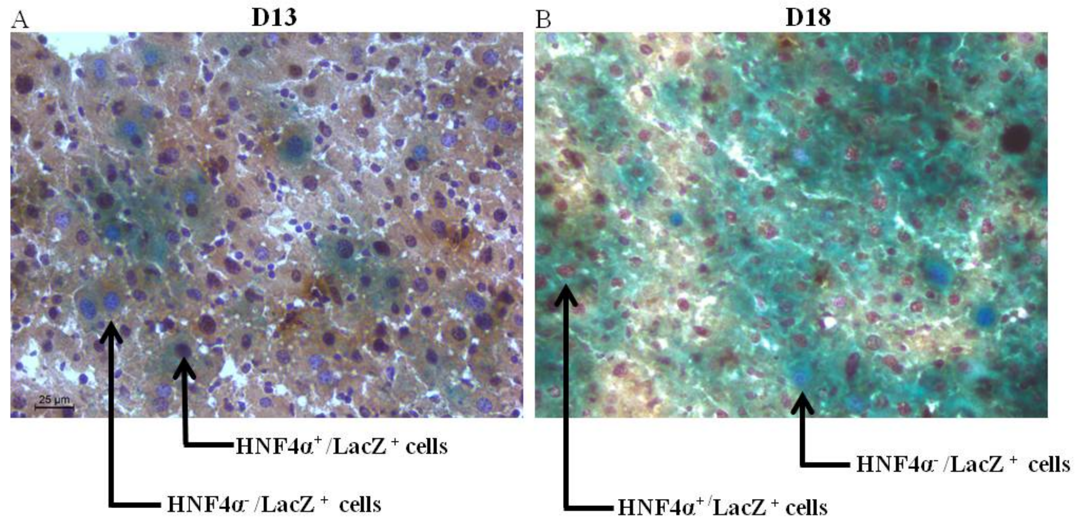

Figure 8: LacZ and HNF4α co-staining of maternal livers in pregnant mice. Pregnant mice were injected with tamoxifen on gestation days 10, 11, and 12 (intraperitoneal, once each day). Gestation day 13 (D13) and 18 (D18) maternal livers were frozen in OCT cooled with heptane on dry ice. Ten micrometer thick maternal liver sections were prepared and stained for the LacZ enzyme and HNF4α. Note that both D13 (**A**) and D18 (**B**) maternal livers showed two distinct populations of LacZ-expressing cells: HNF4α-positive and HNF4α-negative, LacZ-expressing cells. D18 maternal livers showed greater numbers of HNF4α-positive LacZ-expressing cells compared to HNF4α-negative, LacZ-expressing cells. Note: LacZ-expressing cells are blue in colour.

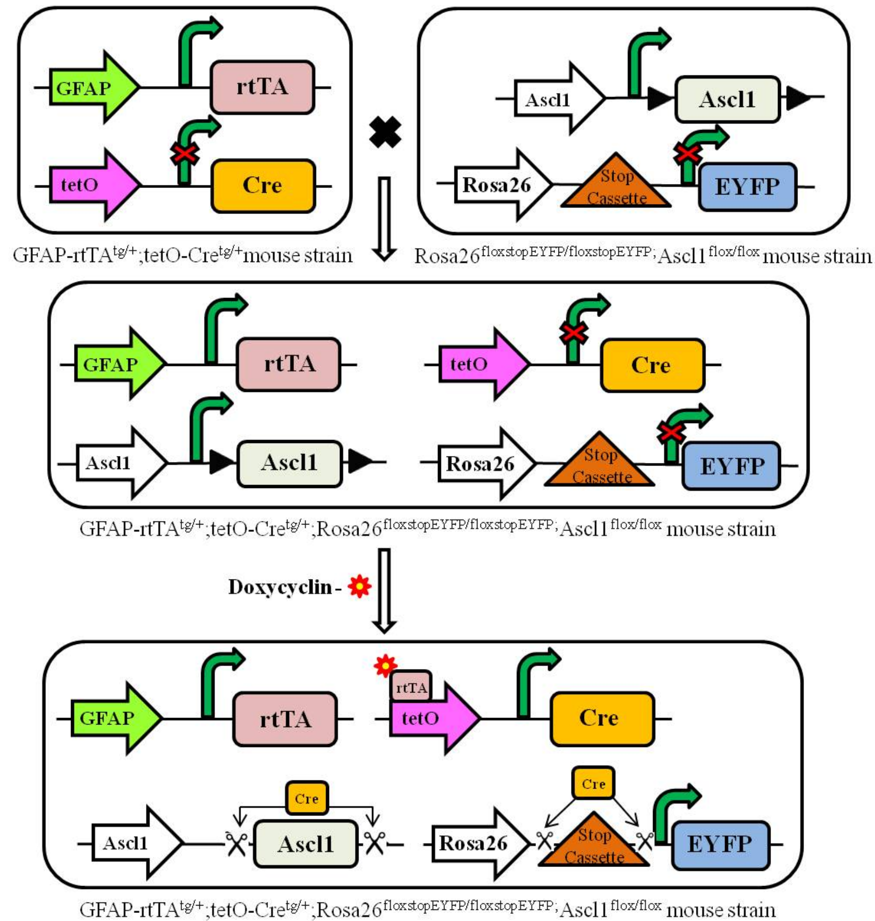

Figure 9: Generation of the GFAP-rtTA$^{tg/+}$;tetO-Cre$^{tg/+}$; Rosa26$^{floxstopEYFP/floxstopEYFP}$;Ascl1$^{flox/flox}$ conditional knockout (cKO) mouse model. This mouse model was generated by serially mating the GFAP-rtTA$^{tg/+}$, the tetO-Cre$^{tg/+}$, and the Rosa26$^{floxstopEYFP/floxstopEYFP}$;Ascl1$^{flox/flox}$ mice. When cKO mice are treated with doxycycline, nuclear translocation of rtTA occurs in GFAP positive hepatic stellate cells. rtTA activates the tetO promoter, which drives expression of Cre recombinase enzyme. Cre recombinase enzyme then floxes out the stop cassette and the *Ascl1* gene. Thus, *Ascl1* is conditionally knocked out from hepatic stellate cells while simultaneously labeling them with EYFP.

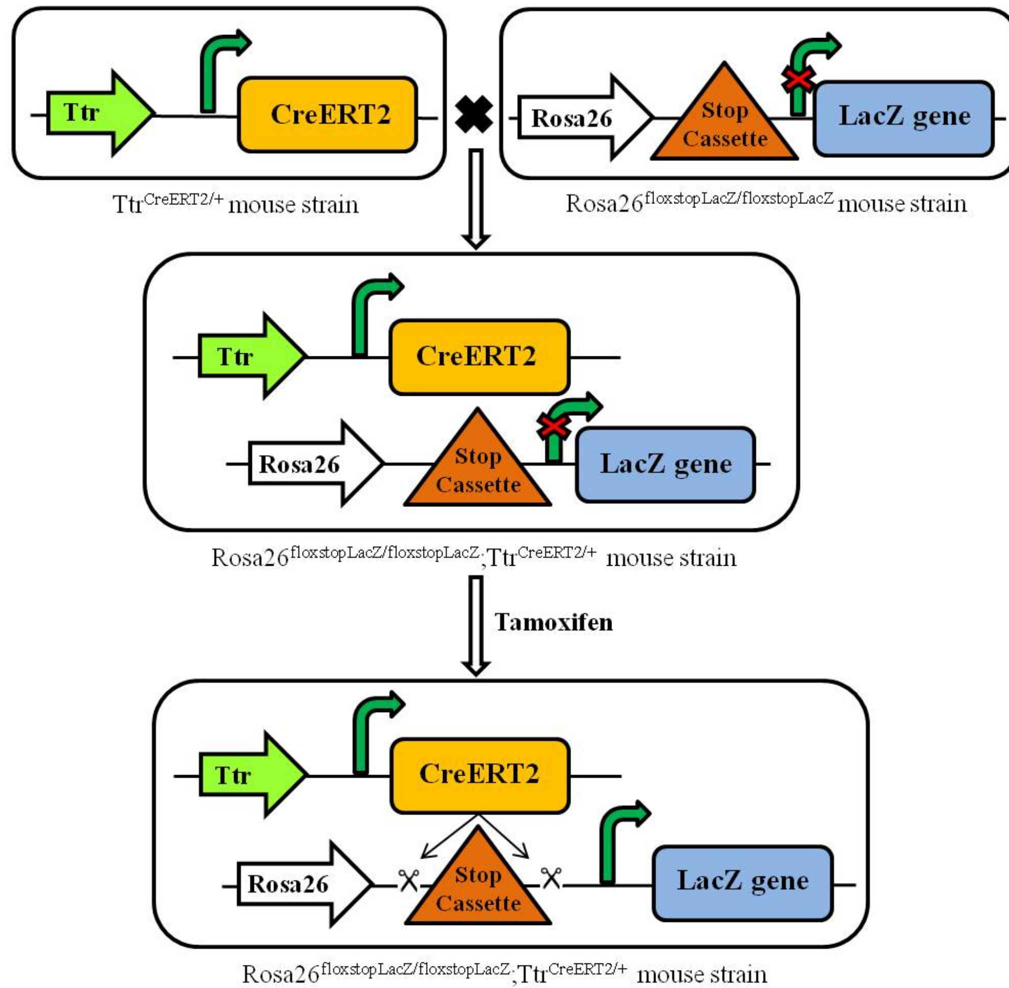

Figure 10: Generation of Rosa26$^{floxstopLacZ/floxstopLacZ}$;Ttr$^{CreERT2/+}$ mouse model. This mouse model was generated by mating Rosa26$^{floxstopLacZ/floxstopLacZ}$ mice with Ttr$^{CreERT2/+}$ mice. When the Rosa26$^{floxstopLacZ/floxstopLacZ}$;Ttr$^{CreERT2/+}$ mice are treated with tamoxifen the CreERT2 fusion protein, expressed in Ttr-expressing hepatocytes, is translocated into the nucleus. Inside the nucleus the Cre recombinase enzyme floxes the stop cassette of the Rosa26^{floxstopLacZ} transgene. Hence, all *Ascl1*-expressing cells are labeled with LacZ enzyme permanently.